Investigating MCM loading during quiescence and cell cycle re-entry

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Abstract

Before DNA replication, Minichromosome Maintenance (MCM) complexes are loaded on DNA to unwind it, allowing replication to proceed. This loading process is facilitated by two proteins, Cdc6 and Cdt1, and the origin recognition complex (ORC). When cells exit the cell cycle, thereby entering a quiescent state, MCM loading stops and therefore DNA replication cannot occur. We noticed that during the quiescent state, Cdc6 and Cdt1 are downregulated while ORC is still present. P38 is also known to inhibit MCM loading in some cases and might be causing slow MCM loading as well. To investigate MCM loading in quiescent cells and cells just coming out of quiescent state, we first constructed modified cells that produce the two factors during quiescent state through cloning. Then, we probed for MCM loaded on chromatin through a combination of chromatin fractionation and immunoblotting. Our results showed that expressing Cdt1 and Cdc6 while inhibiting p38 in quiescent cells cannot achieve MCM loading and thus besides the absence of these proteins, there are other unknown factors inhibiting MCM loading. Inhibiting p38 does not speed up MCM loading as well. These results suggest the existence of new factors or mechanisms on the transition between quiescent and proliferating cells. Further explorations on them can be important in understanding wound healing and cancer development.

Introduction

Proliferating eukaryotic cells replicate their DNA before cell division in order to divide into two daughter cells. DNA replication in proliferating eukaryotic cells is initiated at DNA replication origins located on chromosomes. Before the initiation of DNA synthesis in S phase of the cell cycle, the Minichromosome Maintenance complex (MCM) needs to be loaded on DNA in G1 phase. MCM is an essential factor of the DNA helicase that unwinds DNA at the beginning of DNA replication. The loading process is facilitated by origin recognition complex (ORC) and two proteins Cdt1 and Cdc6. After ORC 1-6 bind the origin of replication, Cdc6 is recruited which further recruits Cdt1. Cdt1 then brings MCM 2-7 hexamer to the origin of replication and loads the hexamer onto DNA through binding to Cdc6 and ORC and ATP hydrolysis (Bryant, J. A.; Aves, S. J. 2011). MCMs, ORC, Cdt1 and Cdc6 together form the pre-replication complex which is activated at the beginning of S phase. In S phase, MCMs travel with replication forks, unwind DNA and are unloaded as replication forks terminate throughout S phase (Maric et al., 2014; Moreno et al., 2014).

Besides going through the cell cycle and proliferate, cells may also choose to enter a quiescent state, also called G0 phase (quiescent and G0 are used interchangeably in this paper). G0 phase is a non-dividing state in which cells do not divide or synthesize DNA. No MCM loading is observed in quiescent cells (Laskey, R. A. & Williams, G. H. 1998). Studies have shown that in G0 cells, Cdt1 and Cdc6 are down regulated as well while ORCs still remain bounded to chromosomes (Madine, 2000). Based on these results, we suspect that the absence of Cdt1 and Cdc6 is one major cause of no MCM loading in quiescent cells and expressing the two proteins in quiescent cells should promote MCM loading. To test this, we conducted immunoblotting on chromatin fractions of modified G0 cells to see whether there is MCM loading when both proteins are present.

From previous studies we found that Cdt1 is a substrate of mitogen-activated protein (MAP) kinases p38 (Chandrasekaran and Cook 2011). P38 is observed to phosphorylate Cdt1 and block MCM loading in cells under osmotic stress (Chandrasekaran and Cook 2011). To further investigate a potential role of p38 in MCM loading, we included assays to test its effect on MCM loading in quiescent cells and MCM loading dynamic in cells reentering G1 from G0. Our results show that expressing Cdt1 and Cdc6 in G0 cells and inhibiting p38 only induces a small amount of MCM chromatin association in quiescent cells. Doing so do not increase the speed of MCM loading in cells coming out of G0 as well. We therefore show that other unknown factors are likely regulating the formation of pre-replication complex in G0 cells.

Methods

Cell Culture

T98G cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2mM L-glutamine and 10% fetal bovine serum (FBS) and incubated in 5% CO_2 at 37 °C. T98g cells were passaged using trypsin every two or three days (whenever cells in plate reach ~80% confluency). Besides wild type T98g cells, T98g cells with doxycycline-inducible Cdt1 construct and Cdc6-degron allele were also cultured. Doxycycline (100/10 ng /ml) and p38 inhibitor (200 µg /ml) were added to some cells depending on experimental setup. Some T98g cells were cultured to confluence and starved in 0.1% FBS media for 72 hours to reach quiescent state. Some cells in G0 were released into G1 by splitting them 1:3 with trypsin to new dishes containing 10% FBS, DMEM, L-glutamine. All cells were collected by trypsinization. PBS was used to quench cells, which were then transferred into microfuge tubes and pelleted at 2000 rps for 3 minutes. Supernatants were removed and the remaining samples were snap-freezed in dry ice and ethanol and then stored at -80C°.

Whole Cell Lysate and Chromatin Fractionation

Each cell sample was thawed in150 µl supplemented CSK buffer (10 mM Pipes-KOH pH 7.0; 100 mM NaCl; 300mM sucrose; 3 mM $MgCl_2$; 0.1 mM AEBSF; 1 µg/ ml pepstatin A; 1 µg/ ml leupeptin; 1 µg/ ml aprotinin; 10 µg/ ml phosvitin; 1 mM β-glycerol phosphate; 1mM Na- orthovanadate; 0.5% triton x-100; 1 mM ATP) and incubated on ice for 20 minutes. 2 µl of sample were removed for Bradford assay. Based on the assay, some amount of sample was removed to fresh tubes as whole cell lysate sample. The rest of the sample was added with supplemented CSK to 1 ml then pelleted in microfuge at 3000 rpm for 5 minutes. Supernatants were removed and sample was suspended in 1 ml supplemented CSK. After 10 minutes incubating on ice, the sample was pelleted two times using 1ml supplemented CSK each time. Pellet was then suspended in 30 µl supplemented CSK with 5 mM $CaCl_2$ and 10 units micrococcal nuclease. After 10 minutes of incubation, sample was pelleted and supernatants were collected. Another 30 µl supernatants were collected using the same process. For each whole cell and chromatin sample, 4X SDS buffer and BME were added. All samples were stored in -80°C.

Immunoblotting

Samples were boiled in water for 5 minutes and pelleted in microfuge at full speed for 5 minutes. Based on the Bradford assay, samples with equal amounts of total proteins were loaded on a 10% polyacrylamide gel. Gel was ran in 1X SDS buffer under 120V and then transferred to polyvinylidene difluoride membranes /Nitrocellulose under 110V for 90 minutes in 1X transfer buffer. After blocking in 5% milk / 5% BSA TBST (tris-buffed saline plus Tween 20) for 60 minutes, membranes were applied with primary antibodies (MCM3/ HP1β/Cdc6/ Cdt1) and left overnight at 4°C in 2.5% milk/5% BSA in TBST. Secondary antibodies in 2.5% milk/5% BSA were added to the membrane after three washes of TBST. Enhanced chemiluminescent (ECL) (1:5/1:2) was added after another three washes of TBST. Images were acquired using darkroom technique.

Cloning and Cell line Construction

Done previously by the Cook lab (Matson et al. 2017).

Results

MCM loading does not occur in quiescent cells.

To establish an assay to measure MCM loading in proliferating and quiescent human cells and to show the effectiveness of chromatin fractionation, MCM3 and HP1β were probed in chromatin fractions and whole cell lysates of RPE cells, shown in Figure 1. Chromatin fractions are chromtain bound proteins obtained using chromatin fractionation mentioned in methods. Considerable amount of MCM3 was found in chromatin sample of proliferating asynchronous cells while hardly any MCM3 was found in chromatin fractions of G0 cells. This is expected since no MCM loading occurs in quiescent cells and therefore MCM3 should only be found in in asynchronous cells' chromatin fractions rather than in G0 cells' chromatin fractions. Meanwhile, HP1β, a chromatin bound protein, served as a loading control and was found in all samples. The levels of HP1β in all cell lanes indicated even amount of protein loading. Together the results suggest that the outcome of chromatin fractionation agrees with prediction and therefore it's reliable for further explorations.

Effect of Cdc6-degron and doxycycline inducible Cdt1 allele

Cdc6-deg cells refer to T98g cells with Cdc6-degron allele. The mutated Cdc6 protein does not contain the degron portion that causes it to degrade during quiescence but does contain an additional tag which causes it to migrate slower during gel electrophoresis. As a result, Cdc6-degron activated cells should show a different molecular weight in gel electrophoresis. T98g cells with doxycycline inducible Cdt1 construct can produce Cdt1 when added with doxycycline.

To show the effects of Cdc6-degron and doxycycline inducible Cdt1 allele, Cdc6 and Cdt1 were probed in different T98g samples. Shown in Figure 2A, significant amount of Cdt1 was found only in + dox whole cell lanes of T98g quiescent cells. Cdt1 is thought to be not expressed in G0 and should not be found in normal G0 cells. Therefore this show that adding doxycycline can cause G0 cells to regain Cdt1. Both Cdc6-deg and wild type were tested as control.

In figureB2, lanes of Cdc6-deg cells indeed display a shift in Cdc6 signals, suggesting the transfected Cdc6-degron allele is capable of producing G0 stable Cdc6 proteins as expected. Different amounts of doxycycline were added as control, showing that different amounts of doxycycline do not affect the expression of Cdc6 or Cdc6-deg.

No significant amount of MCM loading in G0 cells with Cdc6 and Cdt1 present and p38 inhibited

P38 is also known to inhibit MCM loading in cells with osmotic stress (Chandrasekaran and Cook 2011). So besides Cdc6 and Cdt1, we decided to test the effect of inhibiting p38 on MCM loading as well. Consequently, we added doxycycline, p38 inhibitor to Cdc6-deg and wild type cells. Chromatin and whole cell samples and controls were then obtained through chromatin fractionation and probed for MCM3 and Hp1β using immunoblotting, shown in Figure 3. In chromatin samples, strong MCM signal is expressed in the asynchronous lane, showing that MCM loading took place in asynchronous cells. Other lanes, which contained G0 chromatins, also show some extent of MCM. However, compared to the amount of MCM in the asynchronous lane, the MCM in G0 chromatins is insignificant. The expression of Hp1β control show that the amounts of proteins loaded between asynchronous and G0 lanes are approximately even, differing much less than the amount of MCM displayed. MCM and Hp1β levels in whole cell lanes serve as controls showing the total amount of MCM has not changed. Altogether, we concluded that adding Cdt1, Cdc6 and p38 inhibitor to G0 cells may cause a small amount of MCM loading. However the amount of MCM loading promoted is insignificant in comparison to the amount of MCM loaded in proliferating cells, suggesting that merely modifying Cdt1, Cdc6 and p38 (assuming p38 inhibitor worked) is insufficient for G0 cells to recover MCM loading and other factors or mechanisms are likely present to prevent MCM loading in G0 cells.

Inhibiting p38 does not speed up MCM loading in cells coming out of G0

To see the effects p38 have on MCM loading in cells coming out of G0, T98g cells with doxycycline-inducible Cdt1 construct and Cdc6-degron allele were treated with 200 and 0 µg/ml p38 inhibitor and synchronized to G0 through serum starvation. The cells were released into G1 and collected at time points of 8 hours, 10 hours and 12 hours after release. MCM3 and Hp1β were probed in chromatin and whole cell lysates in the collected cells shown in figure 4. Even levels of Hp1β in each lane suggests equal loading while the gradual increase in MCM3 levels in chromatin demonstrates the process of MCM loading. Comparing the amount of MCM3 in lanes with or without p38 inhibitor, we thought that inhibiting p38 does not greatly increase the speed of MCM loading. The total amount of MCM has not changed as shown in whole cell lanes.

Discussion

We confirm that MCM3 is not seen in chromatin fractions from quiescent T98g cells. This agrees with the expectation that no MCM loading occurs in quiescent cells. We also show that Hp1β, a heterochromatin protein, is found in all chromatin samples which proves the eligibility of chromatin fractionation used in experiments. We also show that Cdt1 and modified Cdc6 are found in quiescent T98g cells having doxycycline inducible Cdt1 construct and Cdc6 degron allele. This provides evidence on functions of both

alleles and suggests that we manage to express both proteins in cells in Go where both proteins are normally downregulated.

We further demonstrate that MCM3 chromatin association is not significantly induced in quiescent T98g cells having both proteins and p38 inhibitor. Comparing to the asynchronous T98g cells, quiescent T98g cells with all sorts of modifications (+/- Cdt1, +/- Cdc6-deg, +/- p38 inhibitor) have much less MCM3 associated with chromatin. We can argue that the modifications have some effect since there seems to be more MCM3 on chromatin from modified cells compared to the negative control. Note that the MCM3 signals in chromatin fractions are partly due to a small portion of non-G0 cells in the samples. The synchronization of cells by serum starvation is not perfect in many cases. Together we conclude that expressing Cdt1 and Cdc6 and inhibiting p38 in quiescent cells cannot fully achieve MCM loading. This result seems to contradict previous studies showing Cdc6 can induce MCM chromatin association (Cook et al.2001). Note that we do see an induce effect in MCM chromatin association as discussed before. However we were expecting to see much more since we are adding both Cdt1 and Cdc6 and we know that ORC remains on chromatin in G0 phase (Madine, 2000). Thus our result shows that factors other than Cdt1 and Cdc6 are likely causing no MCM loading in G0. We believe p38 is a candidate so p38 inhibitor is tested as well but not much has changed. As a result, new factors related to MCM loading mechanism, such as OCTUB 1, should be explored in future studies.

Studies have shown that cells coming out of G0 loads MCMs slowly in their first G1 phase and are thus hypersensitive to replication stress (Matson et al 2019). Since p38 can contribute to stress-induced inhibition of MCM loading, we want to see the effects p38 have on MCM loading dynamics in cells just coming out of G0. We find that inhibiting p38 does not significantly speed up MCM loading in the first G1. This suggest p38 might not be or at least not the only factor causing slow MCM loading. This is not surprising as the difference can be caused by many other things and it's unlikely that p38 is responsible on its own. Again the result encourages us to explore other factors such as Rb phosphorylation and CDK2 location involved in the cell cycle.

One caveat in this study worth noting is the missing of p38 inhibitor control. Since the results from p38 inhibitor are all negative results, it is necessary to have a p38 inhibitor control to show the inhibitor is actually working. The drug has been proved useful in previous studies (Chandrasekaran and Cook 2011) and the control for it hopefully will exist in future studies.

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Figures:

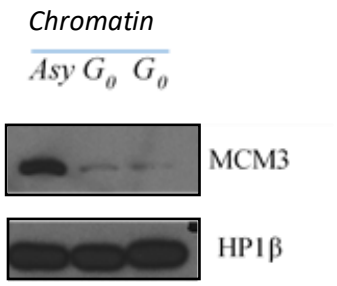

Figure 1. MCM loading does not occur in quiescent cells. Chromatin fractions of asynchronized T98g cells and T98g cells synchronized in G_0 by serum deprivation were collected. Chromatin fractions were then probed for MCM3 and Hp1β by immunoblotting.

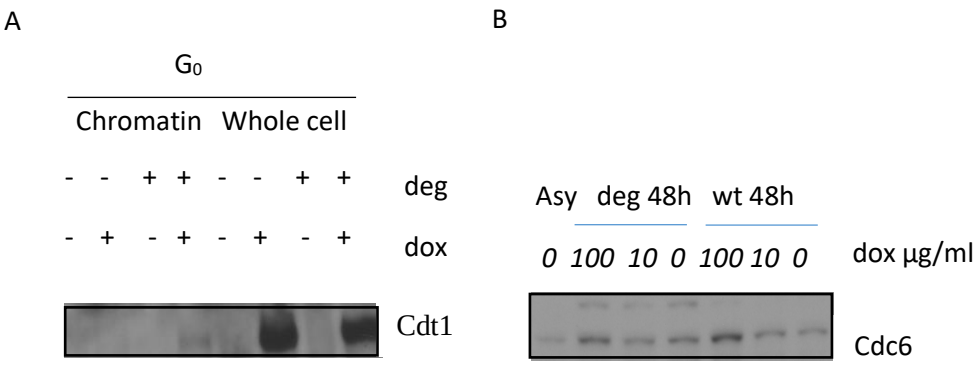

Figure 2, Cdt1 over expressed by doxycycline in cells with doxycycline-inducible Cdt1 construct; cells with Cdc6-degron allele produce mutated Cdc6. (A) G_0 T98g cells with doxycycline-inducible Cdt1 construct and with/without Cdc6-degron allele were treated with 100/0 µg/ml doxycycline. Their Cdt1 levels in whole cell lysates and chromatin fractions were analyzed by immunoblotting. (B) G_0 T98g cells with doxycycline-inducible Cdt1 construct and with/without Cdc6-degron allele were treated with 100/10/0 µg/ml doxycycline and released from quiescent for 48 hours. Cdc6 levels in these cells and wild type asynchronized T98g cells were analyzed by immunoblotting.

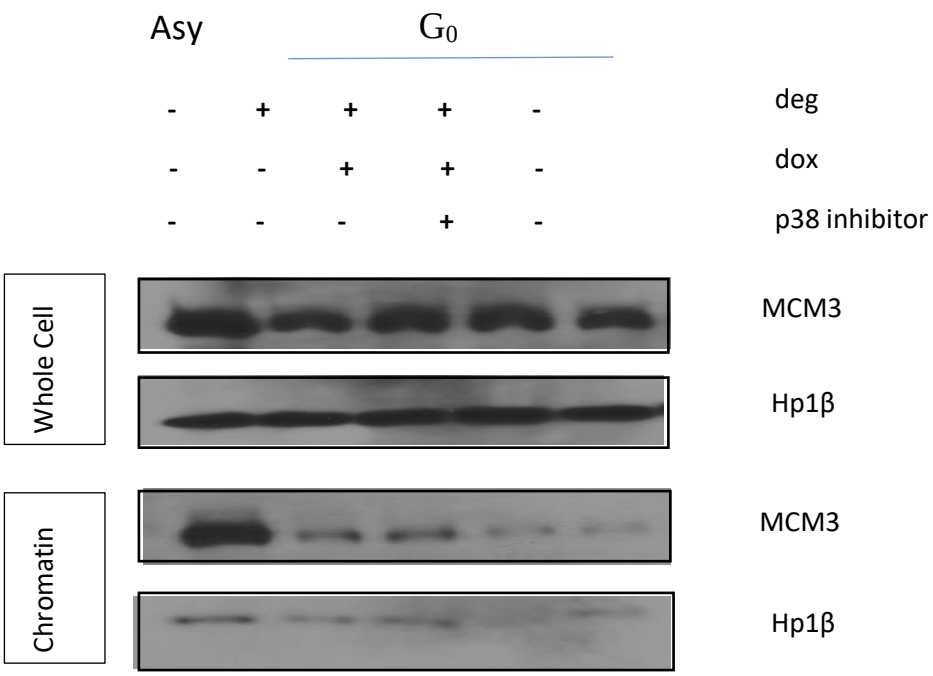

Figure 3. After modifying Cdt1, Cdc6 and p38, no significant amount of MCM loading is found in quiescent cells. G_0 T98g cells with doxycycline-inducible Cdt1 construct and with/without Cdc6-degron allele were treated with 100/0 µg/ml doxycycline and 200/0 µg/ml p38 inhibitor. Whole cell lysate and chromatin of G_0 and asynchronized cells were probed for MCM3 and Hp1β.

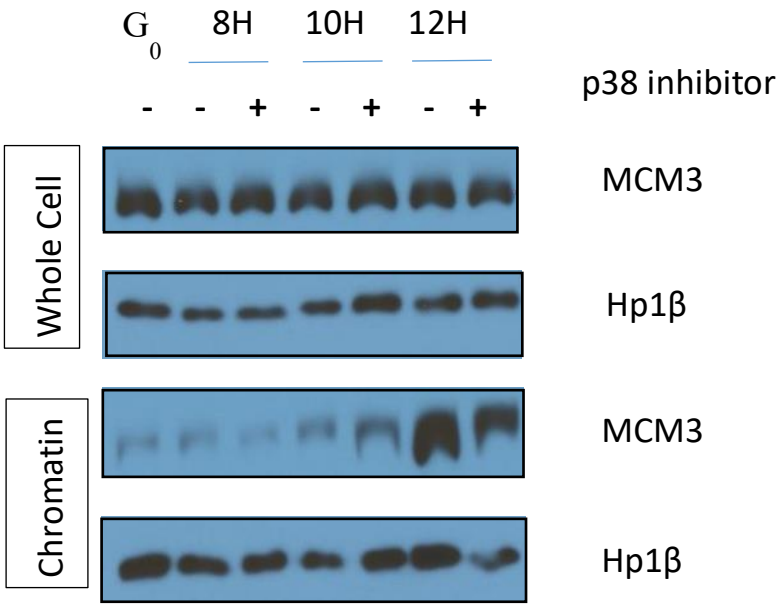

Figure 4. Inhibiting p38 does not speed up MCM loading in cells coming out of G_0.T98g cells with doxycycline-inducible Cdt1 construct and Cdc6-degron allele were treated with 200/0 µg/ml p38 inhibitor and were released from quiescence and collected at different time points. MCM3 and Hp1β were probed in whole cell lysate and chromatin of the collected time point samples.